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Hepatitis B Virus Reactivation in Patients Undergoing Cytotoxic Chemotherapy for Solid Tumours: Precore/Core Mutations May Play an Important Role

Joyce L. Steinberg,[1] Winnie Yeo,[1]* Sheng Zhong,[1] John Y.H. Chan,[1] John S. Tam,[2] Paul K.S. Chan,[2] Nancy W.Y. Leung ,[3] and Philip J. Johnson[1]

[1]*Department of Clinical Oncology, Sir Y.K. Pao Centre for Cancer, Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, Hong Kong*

[2]*Department of Microbiology, Sir Y.K. Pao Centre for Cancer, Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, Hong Kong*

[3]*Department of Medicine, Sir Y.K. Pao Centre for Cancer, Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, Hong Kong*

Reactivation of the hepatitis B virus (HBV) is a rare, but well described complication of cytotoxic chemotherapy that may result in hepatic failure. Patients who are chronic carriers of the HBV and who have a G to A mutation at nucleotide 1896 in the precore region may develop more severe liver disease, possibly because of rapid selection and enhanced replication ability of the mutant strain. Such mutant viruses have been implicated occasionally in chemotherapy induced reactivation of hepatitis B virus. In this report, 5 patients with solid tumours were identified to have developed severe hepatitis B virus related liver disease during treatment with cytotoxic agents (with dexamethasone as antiemetic). All had clinical and serological evidence of reactivation of the HBV. Three patients developed icteric hepatitis; 2 fully recovered, and 1 had died from progressive metastatic disease while recovering from the reactivation. The other two died from progressive liver failure. Direct sequencing of the polymerase chain reaction (PCR) products of the precore (preC) and precore promoter region of the HBV-DNA was carried out on the patients' serum samples taken during the episode of reactivation. In each case, similar mutations (G to A) in nucleotide 1896 of the preC region were found, together with additional mutations in the preC promoter. The present findings suggest that reactivation involving a mutant hepatitis B virus may lead to liver failure, which is possibly more severe than that caused by wild type HBV, and can be triggered by cytotoxic chemotherapy, or the administration of corticosteroids. In Eastern Asia the HBV carriage rate in adults is high. HBV reactivation and severe liver disease during cytotoxic treatment may become a serious and common problem in this region as cytotoxic chemotherapy is more widely used. Patients should be screened routinely for HBsAg in endemic areas of chronic hepatitis B virus infection prior to receiving cytotoxic treatment. The possibility of HBV reactivation should be considered in patients developing liver dysfunction. Patients who are HBeAg negative/Anti-HBe positive, and are suspected to be having an HBV reactivation, should have HBV-DNA levels measured for confirmation as they may carry a mutant HBV. ***J. Med. Virol. 60: 249–255, 2000.*** © 2000 Wiley-Liss, Inc.

KEY WORDS: HBV; immunosuppressive treatment; mutant; reactivation; liver disease

INTRODUCTION

Reactivation of the hepatitis B virus (HBV) is a well-recognized complication of cytotoxic chemotherapy for malignant disease. Most reported cases have involved hematological malignancies, but occasional cases have been described in patients with solid tumours [Galbraith et al., 1975; Hoofnagle et al., 1982; Thung et al., 1985; Bird et al., 1989; Lau et al., 1989; Liang et al.,

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*Correspondence to: Winnie Yeo, Department of Clinical Oncology, Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, Hong Kong.

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1990; Lok et al., 1991; Pinto et al., 1990; Soh et al., 1992; Wong et al., 1996]. The reactivation is characterized by high levels of serum HBV-DNA, abnormal liver function tests (LFTs) and clinical hepatitis ranging in severity from anicteric hepatitis to severe, progressive hepatic failure which results in death. In a study of lymphoma patients, 21% of HBsAg positive patients had reactivation of HBV during treatment and severe progressive hepatic failure was reported in 6 of 22 of these patients [Liang et al., 1990].

The mechanism of reactivation and the associated liver damage is incompletely understood. It has been postulated that, following cessation of cytotoxic chemotherapy, which is immuno- and myelo-suppressive, there is a rebound increase in lymphocyte number which leads to a rapid destruction of infected hepatocytes with consequent severe hepatitis and raised HBV-DNA levels [Hanson et al., 1986; Lok et al., 1991]. This is consistent with the idea that one mechanism of hepatocyte damage in patients with chronic HBV infection is by a host T cell attack in virally infected cells (the core antigen as the main target [Mondelli et al., 1982]).

Mutations in the precore (preC) region of the HBV-DNA have been described in patients with chronic hepatitis. A point mutation in the preC region of the viral DNA, changing from G to A at nucleotide (nt) 1896, produces a stop codon, a truncated HBe antigen, and disappearance of HBeAg [Uy et al., 1986; Carman et al., 1989; Okamoto et al., 1990]. An association of this preC 1896 mutation with severe liver diseases has been described [Carman et al., 1991; Kosaka et al., 1991; Omata et al., 1991; McMillan et al., 1996]. However, only a few cases of preC mutations have been reported in reactivated HBV following cytotoxic chemotherapy. These involved 4 patients with non-Hodgkin's lymphoma (NHL), 1 with prostate cancer patient and 1 with a glioblastoma [Yoshiba et al., 1992; Duclos-Valle et al., 1994; Nishizono et al., 1997].

In this report the HBV-DNA sequences at the preC and the promoter of the preC region of the HBV genome in serum samples from 5 patients were analyzed. These were three patients with breast cancer, 1 patient with a germ cell tumour and 1 patient with small cell lung cancer who developed HBV reactivation and severe liver disease whilst receiving cytotoxic chemotherapy. The promoter region and HBx were also examined because they also appear to be associated with fulminant disease in chronic carriers [Moriyama et al., 1996; Nishizono et al., 1997; Scaglioni et al., 1997].

MATERIALS AND METHODS

Five patients (3 with breast cancer, 1 with a germ cell tumour and 1 with small cell lung cancer), receiving cytotoxic chemotherapy, and dexamethasone as an antiemetic (Table I), were found to have reactivation of HBV. All had normal liver functions prior to initiation of treatment. Two patients (patients 2 and 3) were known carriers of the HBsAg. The other 3 were found to be carriers at the time of reactivation with the absence of IgM anti-HBc. All were found to be HBeAg negative and HBeAb positive.

Investigations for other causes of liver disease included screening for hepatitis A, hepatitis C, delta agent, EBV and CMV were found to be negative. Progressive malignant infiltration by tumour and hepatotoxic drugs were ruled out as contributing to the hepatitis. IgM anti-HBc (Corzyme-M, Abbott Diagnostics) was below the cut-off value for acute infection (and hence regarded as undetectable) in all patients except for patient 4 who was a known HBsAg carrier. All patients had abnormal LFTs and elevated levels of HBV-DNA ranging from 291.3×10^6 to $>4900 \times 10^6$Eq/ml. The diagnosis of HBV reactivation was therefore established.

Patients 1 and 2 with breast cancer had reactivation after receiving 4 cycles of chemotherapy and patient 3 with breast cancer after 5 courses. Patient 4 with a germ cell tumor had HBV reactivation after 2 courses of high dose treatment that followed 3 cycles of conventional chemotherapy. Patient 5 with small cell lung cancer reactivated after receiving 6 courses of cytotoxic treatment.

Patients 1 and 3 died from severe progressive liver failure despite supportive care. Patient 1 was given lamivudine, an oral dideoxynucleoside, late in the course of her reactivation as it was not readily available at the time of her illness. Patient 2 recovered fully, she received only supportive care because lamivudine was not yet available at the time of her reactivation. Patients 4 and 5 received lamivudine as soon as HBV reactivation was suspected. Patient 4 has fully recovered. Patient 5 had been recovering from the HBV reactivation, but died from progressive metastatic disease.

Serum HBV Status

HBsAg, HBeAg, and anti-HBe, were tested in serum by commercially available immunoassay kits (Abbott,). HBV-DNA was assayed using branched-DNA hybridization assay (Quantiplex™ HBV DNA Assay, Chirion, Emeryville, CA). HAV-IgM was determined using IgMmu capture enzyme immunoassay (Abbott, CA). HCV was tested using anti-HCV IgG enzyme immunoassay (Detect-HCV™ (V.3), BioChem ImmunoSystems Inc., Quebec, Canada).

Sample Preparation and Polymerase Chain Reaction (PCR)

Serum samples were collected and stored at –20° C. Viral DNA was prepared using the proteinase K- phenol extraction method as described. In brief, 200l serum was incubated at 60°C for 4 hr in 20mM Tris-HCl (pH 8.0), 10 mM EDTA, 0. 1% SDS, and 0.8 mg/ml proteinase K. The DNA was extracted with phenol/chloroform and precipitated with ethanol using 20 μg tRNA as carrier. The HBV-DNA were amplified by polymerase chain reaction (PCR) from sera of the five patients. The oligonucleotides were synthesized by Oligos Etc. Inc., Cascade. A fragment of 370 basepairs (bp)

TABLE I. Details of the Five Cancer Patients With HBV Reactivation During Chemotherapy*

Patient	1	2	3	4	5
Primary cancer	Breast cancer	Breast cancer	Breast cancer	Yolk sac tumour of mediastinum	Small cell lung cancer
Stage of cancer	T2N2M1	T1N1M0	T3N1M1	III extragonadal germ cell tumour	Limited
Cytotoxic drugs	AC	AC	AC	BEP → HD	CAV & XRT
Dexamethasone	Yes	Yes	Yes	Yes	Yes
HBV status prior to chemotherapy	unknown	HBsAg+	HBsAg+	unknown	unknown
No. of cycles received before reactivation	4	4	5	3 BEP+ 2HD	6
LFTs prior to chemotherapy	Normal	Normal	Normal	Normal	Normal
Peak ALT/Tbil	7320/301	3834/30	781/165	572/3321	93/832
HBsAg	+	+	+	+	+
IgM anti-HBc	–	–	–	–	–
HBeAg	–	–	–	–	–
Anti-HBe	+	+	+	+	+
HBV-DNA level (Eq/ml)	$>4900 \times 10^6$	291.3×10^6	$>4900 \times 10^6$	2190×10^6	$>4900 \times 10^6$
Lamivudine given	+ (late in course)	–	–	+ (early in course)	+ (early in course)
Outcome	Died	Recovered	Died	Recovered	Died[a]
PreC 1896 mutation	+	+	+	+	+
PreC promoter 1799 mutation	+	+	+	+	+
PreC promoter additional mutation	+	–	+	+	+
HBx mutations	+	–	+	+	+

*BEP: bleomycin/etoposide/cisplatin; AC: doxorubicin/cyclophosphamide; LFTs: liver function tests; Tbil: total bilirubin; CAV: cyclophosphamide/doxorubicin/vincristine; HD: high dose bleomycin/etoposide/cyclophosphamide; XRT: radiation therapy.
[a]died of metastatic disease.

corresponding to nt 1619-1978 was amplified with nested primers: outer primers: 5′-GTTCACGGTGGTCTCCATG-3′ (nts 1609-1627) and 5′-AGTGCGAATCCACACTC-3′(nts 2286-2270); inner primers: 5′- CATGGAGACCACCGTGAAC-3′ (nts 1609-1627) and 5′-TGCCAAGTGTTTGCTGACGC-3′ (nts 1978-1960), which included HBV X gene, preC promoter, preC gene of the completed genomic (–) DNA strand (Accession no: M38454, NID: g329616) [Gan et al., 1987]. Nucleotides are numbered from the putative *EcoRI* site in HBV-DNA [Galibert et al., 1979]. PCR was performed under the following conditions: Briefly, 50 μl reaction mixture containing 2 μl extracted serum viral DNA (equal to 10 μl serum), 50 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTPs, 0.01% gelatin, and 1.5U *Taq* DNA polymerase (BRL, USA). The first round reaction was hot started, with the cycling parameters being 1 min at 94°C, 50 sec at 57°C, and 2 min at 72°C for 35 cycles. The PCR parameters for the second round were exactly the same as the first round.

DNA Sequencing and Mutation Identification

The amplified product after the second round of PCR was purified from agarose gel using Sephaglas™ BandPrep Kit (Pharmacia Biotech, Uppsala, Sweden). The amplified DNA was then sequenced following the protocol supplied with the Amersham Bio-Science cyclist DNA sequencing kit (Amersham, Life Science Inc., Cleveland, OH). The primers used for sequencing the precore promoter and precore/core region were the inner primers for PCR. Mutations were confirmed by repeat PCR and sequencing analysis. The HBV sequences were determined and compared with the published HBV sequences of the dominant HBV sequences in this region [Gan et al., 1987; Okamoto et al., 1988]. Any nucleotide or amino acid differing from the respective genotype consensus sequence, but which was common in other genotypes, was not considered to be a significant mutation.

RESULTS

The PCR products of the precore/core region of the HBV genome amplified by PCR from the sera of the patients were sequenced. The HBV genomes from all five patients contained the precore/core G-to-A mutation at nt 1896. In addition, the sample from patient 2 also had a G-to-A mutation at nt 1899. These mutations are well documented and they create a stop codon at codon 28 of the precore open reading frame (ORF) which results in the production of a truncated protein.

To explore whether there were mutations at the basal promoter of the HBV preC region which may affect viral behavior, the promoter region of the preC gene was also sequenced. In total, 14 nt mutations were identified (nt 1741 to 1816). In patient 1, there

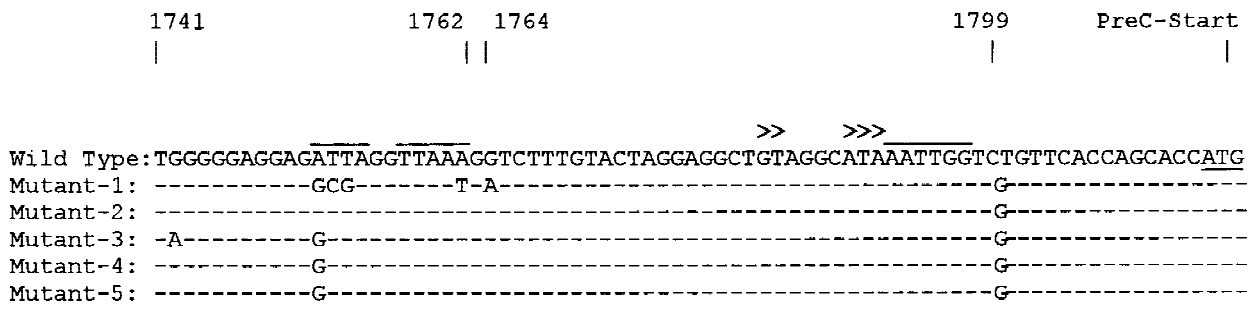

Fig. 1. DNA sequence of the promoter region of preC/C. The nucleotide sequences of the reactivated HBV DNA from the five patients with solid tumors are shown indicating the various mutations in the preC/C promoter.

were six mutations at nt 1752 (A to G), 1753 (T to C), 1754 (T to G), 1762 (A to T), 1764 (G to A) and 1799 (G to A), respectively. In patient 2, there was a C to G mutation at nt 1799. In patient 3, there were three mutations at nt 1742, 1752 and 1799 changing nucleotide from G to A, A to G and C to G, respectively. In patients 4 and 5, there were mutations at nt 1752 (A to G) and nt 1799 (C to G). None of these mutations lie within the essential sequence for the pre-genomic RNA (nt 1788 to 1795). Four of the patients (patient 1, 3, 4, and 5) have had mutations at the A-T rich region of the preC/C promoter (Fig. 1). No mutations were found at the transcriptional initiation sites for the precore and genomic RNA, which are located at nts 1792 ± 1 and 1821 ± 2, respectively.

These mutations at the promoter region of preC also led to amino acid changes in the carboxyl-terminal part of the HBx protein in patients 1, 3, 4, and 5. The HBx at codon 127 from patient 1 contained an Ala while that of patient 3, 4, and 5 contained a Val replacing Ile. At codon 130 and 131 of HBX gene, patient 1 had two additional mutations changing a Lys and a Val into a Met and an Ile respectively (Fig. 2).

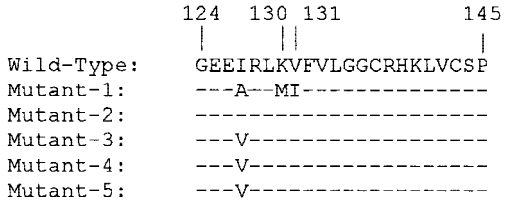

Fig. 2. Amino acid substitutions in the carboxyl-terminal part of the X protein in mutants from the HBV reactivation patients.

DISCUSSION

The 5 patients described here all developed severe liver disease whilst receiving cytotoxic chemotherapy for solid tumours. The clinical picture, together with seropositivity for HBsAg and gross elevations of serum HBV-DNA, in the absence of other causes of liver disease, established the diagnosis of hepatitis B virus reactivation. HBV reactivation, causing death from hepatic failure after cytotoxic treatment, has been well described in patients with non-Hodgkin's lymphoma [Bird et al., 1989; Galbraith et al., 1975; Hoofnagle et al., 1982; Lau et al., 1989; Liang et al., 1990; Lok et al., 1991; Pinto et al., 1990; Soh et al., 1992; Thung et al., 1985; Wong et al., 1996]. In contrast, reactivation in solid tumours has only been occasionally described. There have been only 6 cases of patients with HBV reactivation in which a mutant form of the virus was involved [Yoshiba et al., 1992; Duclos-Valle et al., 1994; Nishizono et al., 1997]. Two of these 6 patients died from severe, progressive hepatic failure and all were, as in the present series, HBeAg negative/Anti-HBe positive, indicating that a mutation was likely to be present in the preC region. In the five patients described in this report, reactivation with HBV precore mutation was confirmed by direct sequencing at nt 1896 which generates a stop codon (TAG), [Uy et al., 1986; Carman et al., 1989; Okamoto et al., 1990] together with mutations in the preC promoter region.

The mechanisms with HBV reactivation during cytotoxic chemotherapy, and the role of these mutant viruses is unclear. It has been proposed that cytotoxic chemotherapy (and in our case including corticosteroids used as an antiemetic) enhance replication of HBV [Scullard et al., 1981; Rakela et al., 1983; Chou et al., 1992; Cheng 1996]. Liver damage, after cessation of treatment, may then be caused by a rebound recovery of immune function which attacks the infected hepatocytes [Mondelli et al., 1982; Alexander et al., 1983; Alexander et al., 1986; Hanson et al., 1986]. On the basis of the present observations, mutant viruses appear to lead to severe reactivation. Three possible mechanisms are proposed. First, the mutations in the preC promoter region, may lead to increased HBcAg production and enhanced viral replication [Scaglioni et al., 1997]. The increased viral replication may increase the number of hepatocytes infected and, since the main targets for cytotoxic T-cells are the cytoplasmic HBcAg peptides [Mondelli et al., 1982; Moriyama et al., 1996], the increased HBcAg may enhance the immune attack against the hepatocytes, thereby leading to a more severe hepatitis. Second, the precore promoter mutations, and the precore mutation (which often occur together [Scaglioni et al., 1997]) decrease or eliminate expression of HBeAg. HBeAg is also a target for both cytotoxic T lymphocytes and antibody dependent cellular cytotoxicity. HBeAg circulates in the blood [Milich et al., 1990]. HBeAg and HBcAg have been found to

have cross reactivity. Both are targets for cytotoxic T lymphocytes (CTL) and antibody dependent cellular cytotoxicity (ADCC) [Milich et al., 1988]. Normally, HBeAg is a toleragen because it blunts the effect of CTL and ADCC against the HBcAg on the hepatocyte. In the absence of the HBeAg, there is an escalated immune response against the HBcAg resulting in a more severe hepatitis [Milich et al., 1990]. Third, the peptide, produced as a result of the stop codon from mutation at nt 1896, in itself may be toxic to the hepatocytes [Dienes et al., 1995].

The mutations at the preC promoter also affected the amino acid sequences of the overlapping HBx gene of patients 1, 3, 4 and 5. The function of the X gene product is unclear but it may upregulate HBV replication. Hence, these mutations might also contribute to the pathologic role of HBV and the severity of disease [Yen, 1996].

In patients having an acute HBV infection with the preC stop codon mutation at nt 1896, a fulminant course has been reported in most [Omata et al., 1991; Kosaka et al., 1991; Carman et al., 1991], but not all patients ([Mphahlele et al., 1997]. The 5 patients reported in the present study were, however, likely to have been chronic carriers because they lived in an endemic area, had no history of a prodromal illness and had normal LFTs prior to commencing chemotherapy. In addition, IgM anti-HBc antibodies, which have been known to be detectable in chronic as well as acute HBV infections [Colloredo et al., 1996], were undetectable in 4 of the 5 patients using the existing assay (Corzyme-M, Abbott Diagnostics). This may have been due to the patients' inability to mount the appropriate antibody response in an acute infection during immunosuppression induced by chemotherapy. It is more likely, however, to be due to the sensitivity of the assay used, which has been set to discriminate between acute and non-acute infections [Diment, 1991; Gaeta et al., 1995; Colloredo et al., 1996]. In chronic HBV infection, the mutation at nt 1896 has also been linked to more severe liver disease in chronic carriers in some but not all reports [Carman et al., 1989; Okamoto et al., 1990; Omata et al., 1991; Brunetto et al., 1990; Bonino et al., 1986; Naoumav et al., 1992; Tur-Kaspa et al., 1992; Chan et al., 1998].

Although reactivation is classically described after cessation of chemotherapy, in all our patients it developed during the course of chemotherapy, between the fourth and sixth cycles. We have considered the possibility that the cytotoxic agents may have been directly mutagenic to the virus. However, we did not have serum available from any patients prior to reactivation for sequencing. In a report of patients receiving liver transplantation, HBV-DNA sequencing was undertaken pre- and posttransplant. The sequences were very similar indicating the immune suppression given after transplant had little influence on the viral sequences [McMillan et al., 1996]. However, Nishizono et al. [1997] sequenced the HBV-DNA in patients prior to the commencement of immunosuppressive treatment in asymptomatic HBsAg, anti-HBe positive carriers, during HBV reactivation and in the post-reactivation phases. It was reported that during the asymptomatic period, the dominant mutation was an 8-bp deletion located in the basic core promoter region at nt 1768 to 1775. During and after reactivation, the mutant clone disappeared or decreased in number and the dominant clone possessed a precore stop codon mutation G to A at nt 1896. Other clones possessed additional point mutations A to T at nt 1762, G to A at 1764 and a new point mutation C to T an nt 1653. Nishizono et al concluded that the decrease or disappearance of the mutant clone possessing the 8-bp deletion, involvement of the mutation at nt 1653 and stimulation of the glucocorticoid responsive element may have induced viral propagation and HBV reactivation [Nishizono et al., 1997].

In the present study, five patients experienced icteric hepatitis with derangements seen in their serum transaminase, bilirubin and coagulation profiles. One of these was given supportive care and the abnormalities fully resolved spontaneously. Two others were given lamivudine soon after they were found to be reactivating; of whom one fully recovered and the other died from progressive metastatic disease despite recovering from the reactivation. The other 2 patients died from severe, progressive hepatic failure; one patient was given lamivudine late in her course when it became available, despite HBV-DNA levels quickly becoming undetectable, she still died from progressive liver disease. Lamivudine may have been more effective if it were given earlier in the course of the reactivation or as a prophylactic measure prior to chemotherapy.

As the incidence of many tumours including breast and lung cancer is rising rapidly in Asia [Hong Kong Cancer Registry, 1998], many more people will be exposed to cytotoxic chemotherapy. Up to 15 percent of the normal population are carriers of the hepatitis B virus in Asian countries and the prevalence may be as high as 25% in those with lymphomas [Liang et al., 1990; Lok et al., 1991]. Clearly, patients found to be HBV carriers should be closely monitored during treatment with LFTs and HBV-DNA levels. The present study suggests HBeAg negative precore mutant virus may play a significant role in HBV reactivation in patients receiving immunosuppressive therapy. Clinical trials to establish effective treatment of HBV reactivation during chemotherapy using antiviral therapy such as lamivudine are clearly required. Eliminating corticosteroids as antiemetics in HBsAg carriers may minimize the risk of reactivation [Cheng, 1996]. Prospective studies to assess the impact of mutant viruses in HBV reactivation during cytotoxic chemotherapy are currently undertaken.

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